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TITLE: A Cohort Study of the Relationship Between c-erbB-2 and Cyclin D1 Overexpression, p53 Mutation and/or Protein Accumulation, and Risk of Progression from Benign Breast Disease to Breast Cancer; and Creation of a Bank of Benign Breast Tissue

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Previously, we determined whether c-erbB-2 overexpression and/or p53 protein accumulation with or without gene mutation are molecular markers of increased breast cancer risk. That investigation, which involved 552 subjects, was conducted as a case-control study nested within a cohort of 5,059 women with benign breast disease (BBD) who participated in the National Breast Screening Study (NBSS). The cohort consists of women with histologically-confirmed diagnoses of BBD for whom paraffin blocks are accessible, for whom extensive risk factor data are available, and for whom follow-up status with respect to breast cancer has been determined. Cases are women with benign breast disease who subsequently developed breast cancer. In this study, we propose to: (1)collect paraffin-embedded benign breast material from the remaining 4,507 (that is 5,059-552) cohort members. (2)enlarge our ongoing case-control study with an additional 38 cases (and their controls) which we anticipate will be identified as a result of a linkage of the NBSS database to the National Cancer Incidence Reporting System. (3)examine whether cyclin D1 overexpression determined immmunohistochemically is a molecular marker of risk of progression from BBD to breast cancer. To date, we have updated the data base, completed the cyclin D1 immunostaining of existing blocks, and initiated block collection of the remaining cohort.

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R Kandel Oct 3/97

PI - Signature Date

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INTRODUCTION

Currently, we are determining whether c-erbB-2 overexpression and/or p53 protein accumulation with or without p53 gene mutation are molecular markers of increased breast cancer risk. (That study was funded by the National Cancer Institute of Canada [NCIC].) The investigation, which involves 552 subjects, is being conducted as a case-control study nested within a cohort of 5,059 women with benign breast disease who participated in the National Breast Screening Study (NBSS). The study funded by the U.S. Army Medical Research and Materiel Command will allow us to enlarge the current case-control study and to create a tissue bank for further studies.

The purpose of this study is three-fold. Specifically, we will:

(1) collect paraffin-embedded benign breast material from the remaining 4,507 cohort members who are not part of the ongoing study. This will establish a bank of paraffin-embedded tissue for a cohort of women on whom there is extensive documentation of risk factor information. With further follow-up of the cohort, it will be possible to enlarge the ongoing case-control study, and to undertake additional studies of newly identified molecular markers of risk of progression to breast cancer. In Canada, paraffin blocks of benign tissue must be retained for 10 years, after which it is at the discretion of the individual hospital as to whether they are kept longer. Therefore, if this resource is to be preserved, the blocks must be collected now.

(2) enlarge our ongoing case-control study with an additional 38 cases (and 5 matched controls per case) which we anticipate will be identified as a result of a linkage of the NBSS database to the National Cancer Incidence Reporting System which is scheduled for late 1995. We propose to examine biopsies from these subjects for evidence of c-erbB-2 overexpression, p53 protein accumulation and p53 gene mutation.

Addition of these cases and controls to the previous study will increase its statistical power.

(3) examine whether cyclin D1 overexpression, determined immmunohistochemically, is a molecular marker of risk of progression from BBD to breast cancer in the enlarged case-control study. We hypothesize that cyclin D1 overexpression in benign breast disease is associated with increased risk of progression to breast cancer. Multiple molecular markers are being examined, since progression to cancer probably results from the accumulation of several genetic events.

(1) OVERVIEW OF PROJECTS: We present here a summary of the methods of our ongoing work. Table 1 provides the distribution, by project component and by funding agency, of the study subjects from whom blocks will be requested (please note that these numbers differ from the number of blocks that we expect to obtain).

Table 1. Numbers of subjects by funding agency, component of project, and case/control status

Project Component	NCIC[b]		US Army [a] (ongoing)	
	Cases	Controls	Cases	Controls
Cyclin D1 staining			112	539
C-erbB-2 and p53 analysis	74	349	38	190
Tissue bank		423		4,507

[a] Numbers of blocks requested
[b] National Cancer Institute of Canada

(a) Summary of previous study - We undertook a cohort study of the relationship between p53 and c-erbB-2 overexpression and risk of progression from BBD to breast cancer. The investigation was initiated in July 1994 and is being conducted as a matched case-control study nested within the

cohort of 5,059 women in the Canadian National Breast Screening Study (NBSS) who were diagnosed with BBD. Cases are women with benign breast disease who subsequently developed breast cancer, and 5 controls are matched to each case. Tissue sections prepared from paraffin-embedded tissue are being stained immunohistochemically for c-erbB-2 overexpression and p53 protein accumulation. In addition, the tissue is being analyzed for the presence or absence of a p53 mutation using the polymerase chain reaction followed by single strand conformation polymorphism analysis and transcript sequencing. Odds ratios for the association between the genetic changes and risk of breast cancer will be adjusted for other factors (reproductive, dietary, histological, etc.) which might be related to these changes and which might also influence the risk of progression to breast cancer. These latter factors were measured using self-administered lifestyle and dietary questionnaires.

(b) Summary of ongoing work - We are currently collecting paraffin-embedded benign breast tissue from the remaining 4,507 women in the NBSS (including the 38 cases and 190 controls who will be added to the ongoing investigation - see below) who were diagnosed with BBD. Our approach to collecting this tissue is the same as that employed for the previous investigation. In Canada, benign tissue blocks have to be retained for 10 years, after which it is at the discretion of the individual hospital as to whether they are retained for longer periods of time. Therefore, it is vital that efforts be made to collect the blocks now.

The collection of these blocks will allow us to enlarge the previous study, since we expect to identify an additional 38 cases as a result of the forthcoming linkage (in late 1997) of the NBSS database to the National Cancer Incidence Reporting System. (The corresponding paraffin-embedded malignant breast tissue will be sought.) We will also identify 5 controls for each of these cases. We will examine these additional cases and controls for c-erbB-2 overexpression and p53 protein accumulation and gene mutation. For all subjects in the case-control study nested cohort study, we will determine which of them display immunohistochemical evidence of overexpression of cyclin D1 in their benign breast tissue. Cyclin D1 expression is being

studied because recent evidence suggests a potential role for cyclin D1 in mammary carcinogenesis and because it is likely that multiple genetic events are involved in carcinogenesis.

(2) SUMMARY OF LITERATURE: A history of benign breast disease is associated with a two-fold increase in risk of subsequent breast cancer (1,2). However, risk of breast cancer in women with BBD differs according to the histological characteristics of the BBD - risk is higher for women with atypical proliferative forms of BBD, in whom the relative risk (versus that for women without proliferative disease) is of the order of four, while the relative risk for women with proliferative disease without atypia is about two (2-7). There is some evidence to suggest that there are interactions between atypical hyperplasia and family history of breast cancer (3,4), nulliparity (5), and menopausal status (6) in determining risk. In some studies, women with fibroadenomas or non-proliferative forms of BBD have been observed to have a small increase in risk of breast cancer (2,8,9).

The transition from regulated cell turnover in breast tissue to unregulated proliferation and the development of carcinoma in-situ and subsequent tumor invasion and metastasis appears to result from a variety of genetic events, including oncogene(s) activation, as well as inactivation of tumor suppressor gene(s) (reviewed in 10,11,12). For example, amplification of DNA, with or without overexpression of oncogenes such as erbA, erbB-2, c-myc, H-ras, hst and int 2, has been detected in breast cancers (10,13-15). Cyclin D1 overexpression has also been implicated in the development of breast cancer (16-21). Additionally, changes in the tumor suppressor genes p53 and Rb, and changes in or loss of heterozygosity at the chromosomal loci 11q, 17q, 17p, 1p, 16q, and 13q have been detected (10,11,22), as have mutations in the HRAS1 mini-satellite locus (23).

Recently, the BRCA1 gene was identified and shown to be mutated in kindred with hereditary breast cancer (24). Decreased BRCA1 mRNA expression has been detected in sporadic breast cancer in both the in-situ and invasive forms (24a).

Other factors might also be involved in the development of malignancy: growth factors such as fibroblast growth factor(s), insulin like growth factor 1, epidermal growth factor and the ligands for erbB-2 have been implicated in the transformation of normal epithelium to a malignant phenotype

(reviewed in 10, 25,26); and cellular changes, including changes in steroid hormone receptor levels and cellular proteases, and changes in the levels and types of membrane adhesion molecules (integrins)(25,27,28). It is possible that these interact to maintain cancer growth.

At the later stages of carcinogenesis, increased angiogenesis has also been shown to occur (25,29). As well, lower levels of nm23 (metastasis suppressor gene) mRNA correlate with decreased disease-free and overall survival (10), although the exact role of nm23 in breast cancer has not been elucidated (29a).

The contribution of each of these genetic changes to the development of the malignant phenotype is not clear, because breast cancers exhibit genotypic and phenotypic heterogeneity (30-32). Of the genes mentioned above, p53, c-erbB-2, and cyclin D1 are amongst those which most frequently show alteration in breast cancer (observed frequencies vary from 13% to 46% for p53, 20% to 50% for c-erbB-2, and 40% to 81% for cyclin D1) (10,30,33-35). Alterations in these genes have been shown to be present in the earliest stages of human breast cancer (carcinoma in-situ) (34-37).

Cyclin D1: It has only recently become evident that cyclin D1 might contribute to the development of breast cancer (17-21, 38,39). Cyclin D1 is a member of a family of proteins that is required for cell cycle progression. Cyclin D1 complexes with cyclin-dependent protein kinases (Cdk 2,4, and 5) and passage through the cell cycle is regulated by progressive activation and inactivation of different cyclin-dependent kinases (16,18,39-42). This is a complex process which is regulated in part by the timing of cyclin expression and degradation, and expression of Cdk inhibitors. D type cyclins are expressed during progression from G_1 to S phase of the cell cycle. Cyclin D1 can be induced by estrogens, suggesting a potential mechanism through which estrogens influence the risk of developing breast cancer (18). Transfection of cells with vectors expressing cyclin D1 has shown that over-expression of cyclin D1 accelerates G1/S transition (43,44).

Cyclin D1 deregulation may occur through gene amplification or rearrangement by translocation, or through mRNA overexpression without gene amplification (16,18,20,40). To date, no mutant forms of cyclin D1 have been identified in

breast cancer. Aberrant expression of cyclins, and particularly of cyclin D1, has been implicated in a variety of tumors including breast cancers. Immunohistochemical staining demonstrates cyclin D1 overexpression in up to 81% of invasive breast cancers (34,35,38,45). Cyclin D1 overexpression has also been detected in intraductal carcinoma, and is maintained in the invasive component (21).

Recent experimental studies support the oncogenic potential of cyclin D1. Cyclin D1 amplification has been observed in 25% of breast cancer cell lines (17). Cyclin D1 transfected with the adenovirus E1A oncogene into BRK cells results in cell transformation (20). Mammary hyperplasia and breast cancer develop in transgenic mice carrying the mouse mammary tumor virus long terminal repeat-cyclin D1 construct (46). These latter experiments also suggest a role for cyclin D1 overexpression in mammary epithelial hyperplasia, a histopathological change known to be associated with increased risk of progression to breast cancer (2-7). Immunohistochemical studies have shown that normal human breast epithelium demonstrates, at most, occasional cells which express cyclin D1 (34,35,38,47).

p53: p53 is also involved in regulating progression of cells from the G1 to the S phase of the cell cycle (48). However, in contrast to cyclin D1, wild-type p53 protein prevents cells from progressing into S phase (49). One mechanism by which p53 exerts this effect is by inducing p21, a cyclin-dependent kinase inhibitor (50-55). Other genes with p53 responsive elements which might also be involved in cell proliferation include GADD45 (56) and mdm-2 (57). Alternatively, under the appropriate stimulus, wild-type p53 can also initiate apoptosis (58).

Overexpression of wild-type p53 can cause immortalization of cells (59,60), but loss of function mutations with or without loss of heterozygosity are usually required for tumor formation. In its mutant form, p53 can stimulate proliferation or act as a dominant transforming oncogene, as has been shown by Lane and Benchimol in transfection studies (61). As well, mutant p53 expressed in cell lines lacking p53 can enhance tumorigenic potential in nude mice (62). Cell lines with mutations in the p53 gene show an increased rate of aneuploidy and gene amplification (62a). In experimental studies, mice deficient in p53 are

susceptible to forming tumors (63).

Mutations in the p53 tumor suppressor gene have been linked to a variety of malignancies including those of the breast (12,33,64-66). The observed frequency of these mutations in the sporadic forms of breast cancer has been observed to be as high as 50% (67).

Mutant and wild-type p53 protein half-life can be altered by interaction with the E6 protein from human papillomavirus, or with heat shock protein 70 or mdm-2 (68-72). Additionally, Moll et al. (73) have shown that in some breast cancers, p53 protein is present in the cytoplasm and excluded from the nucleus. This process would prevent the wild-type protein from exerting its regulatory effect. Cytoplasmic localization of p53 has been identified in other tumors (74).

The presence of p53 mutations and immunohistochemical detection of p53 protein accumulation have been detected in 16% to 33% of intraductal carcinomas (36,75,76). In addition, there is also a literature to suggest that p53 may play a role in the development of breast cancer. p53 protein accumulation has been demonstrated immunohistochemically in the benign breast tissue of patients with the Li-Fraumeni syndrome, who are at high risk of developing breast cancer (76). Four reports have shown positive immunostaining for p53 in benign breast disease (77-79a), and one has shown gene amplification (80). In the latter study, the DNA was not analyzed for the presence of mutations. Our initial results indicate that p53 protein accumulation can occur in benign breast disease (see part 4(ii)). Mutated p53 and overexpression of wild type p53 occurs in the preneoplastic stage of mouse mammary tumor development (81). p53 has also been detected in precursor lesions of other cancers. For example, p53 accumulation was present in dysplastic squamous epithelium of bronchi in 53% of patients with squamous cell carcinoma of the lung (82), and in 23% of biopsies of Barrett's esophagus (83).

C-erbB-2: c-erbB-2 (neu or HER 2) is a normal cellular gene present on chromosome 17q21 (15). It encodes for a membrane protein (p185) which is tyrosine phosphorylated following interaction with its ligands (15). c-erbB-2 overexpression occurs either through changes in amplification and/or mRNA overexpression, perhaps due to OB2-1 overexpression (10,84).

Several experimental studies have suggested that c-erbB-2 may play a role in the pathogenesis of breast cancer. Di Fiore et al (85) showed that five to tenfold overexpression of normal human c-erbB-2 following transfection resulted in transformation of NIH 3T3 cells. The observation that the activated version of the c-erbB-2 gene is detected in the breast cancer that develops in transgenic mice also suggests that this gene may have a role in mammary tumorigenesis (86,87). In one transgenic strain, high levels of expression of the activated gene in the mammary epithelium resulted in the one-step acquisition of the transformed phenotype (87). Moreover, high levels of the transgene transcript could be detected in the earliest stages of mammary tumour formation, suggesting that no further genetic events were required for the conversion of the primary epithelial cell to the malignant phenotype.

c-erbB-2 overexpression has also been examined in several studies of benign and malignant human breast tissue. Overexpression of the c-erbB-2 gene has been noted in early stage human carcinomas (88) and is most frequently detected in ductal carcinoma in-situ (10, 88-90). Immunohistochemical studies demonstrate c-erbB-2 overexpression more consistently in the in-situ component (approximately 50%) than in the infiltrating malignant cells in breast cancer (approximately 20%) (91,92). Several studies have examined c-erbB-2 overexpression in biopsies of benign breast tissue (89,93-95). Of recent studies, two reported either negative or cytoplasmic staining of epithelial cells (89,93), while two others showed positive immunostaining in between 10% (94) and 25% (95) of cases of atypical epithelial hyperplasia. Tsutsumi et al.(96) observed that the adjacent normal epithelium in some breast cancer resections showed stronger c-erbB-2 immunostaining than the cancer.

Conclusion: Epithelial cancers appear to be the result of an accumulation of multiple genetic events, and as demonstrated by Fearon and Vogelstein (97), the earliest genetic change is maintained during progression to cancer. To date, in relation to breast cancer, cyclin D1 and c-erbB-2 overexpression, and p53 mutations are frequent genetic changes detected. While cyclin D1, c-erbB-2, and p53 appear to play an important part in mammary carcinogenesis, their precise role in this process is unclear. For example, it is unclear whether they are

involved in the initiation of transformation or at a subsequent stage, or whether they are just indicators of increased risk of developing breast cancer.

The preceding human and experimental evidence suggests that cyclin D1 and c-erbB-2 overexpression, and p53 protein accumulation with or without gene mutation, may be present in benign breast disease. Since they may also be present in breast cancer, they might either influence progression from BBD to breast cancer or be markers of increased cancer risk.

BODY

(1) STUDY DESIGN:

Our study uses tissues which had been obtained from the cohort of women enrolled within the National Breast Screening Study (NBSS). In the ensuing paragraphs, we describe the NBSS first, and then present details of our investigations.

(a) The National Breast Screening Study: The NBSS is a multi-centre randomized controlled trial of screening for breast cancer in Canadian women aged 40 to 59 at recruitment (98,99). The study involves 89,835 women who were recruited at 15 screening centres across Canada. Recruitment commenced in 1980 and ended in 1985. Women were eligible to participate in the study if they had no history of breast cancer, were not currently pregnant, and had not had a mammogram in the preceding 12 months.

Women aged 40-49 years were randomized either to have annual mammography plus physical examination, or to have initial physical examination only, and women in both the intervention and the control group were taught breast self-examination. Randomization in the 50-59 year age-group was either to annual mammography plus physical examination, or to annual physical examination alone (women in this arm of the 50-59 year age-group were also taught breast self-examination).

(b) Diagnosis of BBD and breast cancer in the NBSS: At each visit, study participants had a physical examination. For those who were randomized to the intervention group, physical examination was followed by mammography, the films from which were read by a study radiologist who was unaware of the physical examination results. If the examiner or the radiologist reported an abnormality requiring further assessment, a referral was made to a review clinic where the participant was seen by a study surgeon. If, on review, a recommendation was made for biopsy, this recommendation was conveyed to the participant's family physician, and the

participant was contacted and asked to visit her family physician for further management.

Women in both control groups were referred for mammography if either they or their primary care physician discovered an abnormality for which referral was warranted. Staff in each screening centre identified the pathology laboratory in which biopsies were examined, and they obtained slides or blocks for review by a locally designated reference pathologist. Results of the histological review of the biopsies were forwarded to the coordinating centre.

(c) Follow-up in the NBSS: Active follow-up continued until 1988. During this phase of the NBSS (when the study participants underwent the screening schedule corresponding to their allocation, as described in (b) above), there was in each study centre a coordinator (usually a nurse) who had experience in clinic or study management. The coordinators were responsible for ascertaining whether the recommended diagnostic procedures had been carried out and for collecting reports of the surgical and pathological procedures from the institutions where they had been performed. Procedures performed independently of the screening process were identified through annual questionnaires sent to study subjects, and reports of these procedures were then obtained from the relevant institutions. Study records for women who moved out of their original area were transferred to the centre nearest their new residence. Following completion of their screening schedule, direct follow-up stopped for those with no diagnosis of breast cancer. However, until 1988-1990 (depending upon the province) information about new diagnoses of breast cancer was obtained by linkage with the provincial cancer registries (cancer is registered in each province in Canada, and, for Ontario at least, registration is essentially complete (100)). Subsequently, new diagnoses of cancer will be ascertained by linkage to the National Cancer Incidence Reporting System, which is operated by the Canadian Centre for Health Information at Statistics Canada, and consists of registration data reported annually by the provincial registries. A linkage is scheduled for late 1997, and we propose that another linkage take place in late 2000 to yield a further 5 years of follow-up data.

(d) Design of the previous study: That investigation was undertaken within the cohort of 5,059 women within the NBSS who received a histopathologic diagnosis of BBD during the active follow-up phase of the NBSS (98,99). In order to reduce costs substantially while having relatively little impact on the precision of the estimates of association (101), the study was conducted as a case-control study nested within this cohort. Cases are women who subsequently developed breast cancer, while controls are women who had not developed breast cancer by the date of diagnosis of the corresponding case. Five controls were selected for each case, and they were matched to the corresponding case on study arm within the NBSS, screening centre, year of birth, and age at diagnosis of BBD.

(e) Case definition: Cases are women with a history of BBD detected during the course of the NBSS who subsequently developed breast cancer (the median interval between diagnosis of BBD and subsequent breast cancer in this cohort is 1226 days). By this definition, 92 cases were identified by previous linkages. We have collected the benign tissue for 74 cases. As described below, as a result of the forthcoming linkage, we expect to identify an additional 38 cases, and on the basis of our experience to date we would expect to obtain 30 (74/92 x 38) of these cases. This would yield a total of 104 cases for the ongoing case-control study.

The number of additional cases of breast cancer which we expect to be identified as a result of the forthcoming linkage was calculated as follows. To the end of 1987, 820 incident cases had been identified in the NBSS, of which 67 (8.2%) were in the cohort of women with benign breast disease. The linkage to the National Cancer Incidence Reporting System in 1997 will yield follow-up data to the end of 1992, and it is expected (see below) that 1600 breast cancer cases will have occurred in the NBSS by this time (by the end of 1992 the study participants had each accrued an average of approximately 9.4 years of follow-up). Assuming that the proportion of this total number of cases which is in the cohort of women with BBD has remained stable, we would anticipate that the number of women with BBD who had developed breast cancer by the end of 1992 will be 130 (i.e. 1600 x 67/820).

The number of existing cases (and controls), and the number of additional cases that we expect to obtain, are summarized in Table 2 below. [The estimate of 1600 cases of breast cancer in the NBSS by the end of 1992 was derived using standard life table techniques, together with age-specific Canadian cancer incidence rates, and Canadian all-cause mortality rates for women for the years 1985-1986 (102,103). It has been observed previously that in the control groups in the NBSS, the observed age-specific breast cancer incidence rates to the end of 1987 were very similar to those expected from national rates. As expected, an excess of breast cancer was observed in the mammography arms, particularly at first screening, which presumably represents the effect of early detection, and possibly also the effect of detection of minimal lesions which might otherwise never have come to diagnosis. In calculating the expected numbers, the excess occurring in the mammography arms was ignored since there is no way of determining if and when this excess will disappear. Additionally, it is clear that removal of prevalent cases at baseline will have reduced the subsequent incidence rate for some period of time, but it is not easy to allow for this. Nevertheless, this probably makes little difference overall, especially given the large expected number of cases of breast cancer.]

(f) Definition of controls: Controls are women who had not developed breast cancer by (but were alive at) the date of diagnosis of the corresponding case (they will, of course, have a diagnosis of BBD). Since there are no estimates of the likely magnitude of the effects of interest on risk of progression from BBD to breast cancer, we selected 5 controls for each case in order to maximize statistical power. Controls were matched to cases on study arm within the NBSS, screening centre, year of birth, and age at diagnosis of BBD (and implicitly on the interval between diagnosis of BBD and the date of diagnosis of breast cancer in the corresponding case). These matching criteria were chosen either because they are related to breast cancer risk (age, and possibly age at diagnosis of BBD) or because they are related to the risk of disease detection (allocation and screening centre). It is also conceivable that at least some of these factors are related to the exposures of interest.

However, it should be noted that little is known about the "epidemiological" correlates of cyclin D1 and c-erbB-2 overexpression, and p53 protein accumulation. Additionally, the implicit matching on duration of follow-up (as well as age) means that the controls have had the same opportunity (at least, in terms of the elapse of time) to develop breast cancer as the cases.

For each case,the controls were randomly selected from eligible subjects in the subgroup defined by the characteristics of the case.(Procedures for the ascertainment of death in the NBSS, and the verification of the cause of death, have been described in detail elsewhere (99).) To date, we have collected the benign tissue for 349 of the 360 controls (i.e.an average of about 4 controls per case). As described in (e) above, as a result of the forthcoming linkage, we expect to identify an additional 38 cases and to obtain blocks for 30 of them. On the basis of our experience to date we would expect to obtain about 113 of the 150 controls for these 30 cases. This would yield a total of 462 controls for the ongoing case-control study.

Table 2. Numbers of cases and controls anticipated for the case-control study

	Cases	Controls
Blocks received to date	74	349
Additional blocks expected as a result of 1997 linkage	30	113
TOTAL	104	462

(g) Questionnaires: Menstrual and reproductive history, use of oral contraceptives, and dietary factors are thought to be involved in the etiology of BBD (104), and it is possible that some of these factors influence risk of progression from BBD to breast cancer. Therefore, estimates of the association between cyclin D1 and c-erbB-2 overexpression and p53 protein accumulation and risk of breast cancer will, where appropriate, be adjusted for these factors. Information on risk factors for BBD will come from questionnaires completed by the NBSS participants.

At the time of their enrolment in the NBSS, all participants completed a questionnaire which sought identifying information, as well as data on factors such as demographic characteristics, family history of breast cancer, menstrual and reproductive history, use of oral contraceptives and replacement estrogens, and cigarette smoking. Additionally, approximately two-thirds of the 89,835 women enroled in the NBSS completed self-administered diet history questionnaires. The dietary questionnaire was introduced in 1982, at which time some women had already been enrolled in the study (and were not seen again at the screening centres). The diet history contained questions on the frequency of consumption and usual portion size of 86 food items, and also had an open-ended section for describing other food items normally eaten. Photographs of various portion sizes were included in the questionnaire to assist participants to quantify intake. The questionnaire also included questions on current and past height and weight, and on consumption of beer, wine, and spirits. A comparison between the self-administered questionnaire and a full interviewer-administered questionnaire which has been subjected to both validity and reliability testing (105) and used in a number of epidemiologic studies (106) revealed that the two methods give similar results for the major macronutrients, dietary fiber, and vitamin C (107).

(h) Statistical power: This will be calculated according to that described by Breslow and Day (108).

(2) CONDUCT OF THE STUDY:

(a) Coding, data entry, and processing: The lifestyle information is available on the computerized NBSS database. The dietary questionnaires are retrieved from storage, and then coded. The dietary data is entered and validation of data entry will be carried out using an existing data entry system for the remaining cohort. Processing of the dietary data to produce estimates of daily nutrient intake will be performed using a computer programme which has already been developed. The NCIC Epidemiology Unit's standard procedures for quality control will be used for coding and data entry. The dietary information will then be merged with the lifestyle information (and subsequently, with the results of

the molecular analyses) to create the study file.

(b) Collection of paraffin-embedded breast material: For the previous case-control study, we created a database consisting of identifying information, plus details of the location and accession number of the 552 paraffin blocks. This information was used to generate lists for each hospital of the study participants for whom we wished to obtain paraffin blocks. We then wrote to the pathologist-in-chief at the hospital seeking the blocks.

This same approach was used to expand the existing tissue bank. The database was updated to include all 5,059 subjects with a diagnosis of BBD in the NBSS. We propose to collect the blocks of the remaining 4,507 (5,059-552) women over 4 years. For hospitals with over 60 requested blocks we have suggested that Dr. Kandel is willing to go to the hospital to select the appropriate blocks. We are doing this to enhance the response rate, because we suspect that at some hospitals the pathologists are willing to provide blocks but do not have sufficient time to select the appropriate ones.

For the previous study, we collected 423 blocks of benign tissue from the 552 paraffin blocks requested. As a result, we predict that we will obtain 3,454 blocks out of the 4,507 blocks requested.

(c) Histopathological Review: The histological sections from blocks received to enlarge the nested case-control study will be reviewed and classified by Dr. Kandel, according to the criteria developed by Page (109).

(d) Experimental methods: In this section we describe the methodology that was used for the cyclin D1 immunostaining. For completeness we also present details of the immunohistochemical staining for c-erbB-2 and p53, as well as the molecular methodology for the detection of p53 mutations.

(i) Cyclin D1 Expression in Breast Disease:

Since we do not have access to frozen tissue, immunohistochemical staining will be used to detect cyclin D1 overexpression. In contrast to those of other cyclins, the level of cyclin D1 does not fluctuate as much, so that the protein should be easily detected (39). It has been shown that this protein, if present, is stable and does not decline rapidly if there is a delay in tissue fixation (46). The antibody that we have selected works on paraffin-embedded tissue. Several reports have shown that this method is more

sensitive than determining whether the gene is amplified, as dysregulated protein expression can result from changes other than gene amplification (34,35,44). There is a good correlation between immunostaining and Western blot analysis which indicates that the positive immunoreactivity is not a false positive (34). Immunohistochemical staining allows cellular localization of the immunoreactivity, so it will be possible to ensure that the cyclin expression is occurring in breast epithelial cells. In addition, this approach will allow us to determine whether the immunoreactivity is present in the histopathology considered to be associated with increased malignant potential. Breast cancers will also be stained in order to determine whether the expression present in the benign breast disease is maintained in the malignant lesion, or is present in the breast cancer only.

Immunohistochemical staining for cyclin D1: 5um sections are cut from the representative block and placed on aminopropyltriethoxysilane (TESPA, Sigma Chemicals) coated slides. These sections are incubated with antibody reactive with cyclin D1 (monoclonal, dilution 1:2000, no crossreaction with cyclins D2 or D3, Upstate Biotechnology, Lake Placid, NY). This antibody works on paraffin embedded tissue. Immunoreactivity was determined using a biotinylated anti-mouse IgG followed by avidin-biotin peroxidase complex (Elite System, Vector Labs,Burlingame,CA) and 3'-3' diaminobenzidine tetrachloride (DAB kit, Vector Labs). Paraffin-embedded cell blocks of the human breast cancer cell line T47D will serve as a positive control. In order to mimic the conditions of the breast biopsies, the cells are harvested, placed in agar, formalin-fixed and paraffin-embedded. As a negative control, the primary antibody is replaced by PBS.

The presence of nuclear staining and the percentage and distribution of positive cells (focal, multifocal or diffuse) will be noted. The location of the immunostaining for cyclin D1 will be correlated to the location of the p53 and/or c-erbB-2 immunostaining to determine whether they occur in similar sites. To assess the accuracy of the immunoreactivity interpretation, a subgroup of cases (n=25) and controls (n=25) will be reviewed a second time (without knowledge of the initial grading) to provide a measure of the misclassification of immunoreactive status, which will enable estimates of the association between cyclin D1 immunoreactivity and risk of breast cancer to be adjusted for misclassification (110).

(ii) c-erbB-2 Overexpression in Breast Disease:

c-erbB-2 overexpression is detected by immunohistochemical staining of paraffin-embedded tissue (89,90,96,111-113). Immunohistochemical staining eliminates the need for labor intensive molecular analysis of the DNA and RNA. (Both DNA and RNA would have to be analyzed as c-erbB-2 overexpression can occur without gene amplification.) In addition to staining benign breast biospies, we are also staining malignant tissue in order to determine whether the overexpression present in the benign breast disease is maintained in the malignant lesion, or is present in the breast cancer only.

Immunohistochemical assessment of c-erbB-2 overexpression: 5um sections are incubated with antibody reactive with c-erbB-2 (NCL-CB11, Novocastra Lab. Inc. Newcastle upon Tyne, dilution 1/160) overnight at 4°C. NCL-CB11 recognizes an internal epitope in formalin-fixed paraffin-embedded material (113,114). After washing, the sections are incubated with biotinylated goat anti-mouse IgG and reactivity detected using the avidin-biotin peroxidase complex and 3'-3' diaminobenzidine. Immunoreactivity is indicated by membrane and/or cytoplasmic staining of cells and is determined by light microscopy. Positive controls include a cell block made of the human breast cancer cell line SK-Br-3 which overexpresses c-erbB-2. As a negative control, the primary antibody is left out. The assessment of immunoreactivity is determined by one of us (R.K.). A subgroup of cases (n=25) and controls (n=25) will be reviewed as described in (i).

(iii) p53 in Breast Disease:

The breast biopsies are first screened for p53 immuno-reactivity (115-120). Immunopositive biopsies will also be analyzed molecularly to determine if a mutation is present (121-123). The cases of benign breast disease which demonstrate p53 accumulation will be correlated with the immunostaining result for the corresponding cancer. This will allow us to determine whether p53 expression is maintained, lost with disease progression, or arises as a later change.

Immunohistochemical staining for p53: Tissue sections are reacted with monoclonal antibody reactive to both wild-type and mutant forms of p53 (clone DO-7, Novocastra Laboratories Ltd. Newcastle upon Tyne, England) following microwave pretreatment (124). Immunoreactivity is determined using a biotinylated-anti-mouse IgG, followed by avidin-biotin

peroxidase complex and 3'-3' diaminobenzidine tetrachloride. Paraffin-embedded tissue, which has been shown by genetic analysis to contain a p53 mutation, is used as a positive control. The negative control consists of leaving out the primary antibody during the incubation.

A positive reaction is indicated by nuclear staining as determined by light microscopy.The assessment of immunoreactivity is determined by one of us (R.K.). A subgroup of cases (n=25) and controls (n=25) will be reviewed as described previously.

PCR-DNA sequencing for p53 mutations: Detection of p53 mutations utilizes polymerase chain reaction (PCR) followed by detection of mutational differences by single-strand conformation polymorphism (SSCP) (125,126).

To minimize DNA "contamination" from stromal cells, the glandular epithelium (which will contain the tissue with positive immunostaining) is identified in the histological section and microdissected out (121). DNA is extracted from the paraffin-embedded tissue using standard techniques (127). This process requires minimal tissue (approximately 1 to 3 sections) and does not destroy the paraffin block. PCR primers (123-126,128) are used to amplify genomic DNA sequences which are the products subjected to SSCP analysis (125,126). For those tumors in which the DNA exhibits mobility shifts by SSCP analysis in exons 5-9, the presence of a mutation will be confirmed by DNA sequencing.

The DNA will be excised from the gel and sequenced directly without cloning, using a set of nested primers and genomic amplification with transcript sequencing (GAWTS). The latter technique has been used successfully for the analysis of p53 mutations in breast cancers (122). Even if there is some normal DNA present from stromal cells, the mutant DNA should be detectable by SSCP analysis as an altered fragment. The mutation will be detectable on DNA sequencing gels (128). Controls for this analysis include the SK-Br-3 breast cancer cell line which has a p53 mutation at codon 175 (128) and MDA-MB-468 and T47D cell lines with mutations at codons 273 and 194, respectively (65), and cells without a mutation (normal fibroblasts). We shall focus on exons 5 to 9 of the p53 gene, since these regions appear to be mutational "hot spots" (33,129). It is possible that some mutations might be missed as not all mutations result in p53 protein accumulation, but this should be a relatively uncommon occurrence (65). DNA mutations identified in the benign disease will be compared to the DNA obtained from its corresponding cancer, to determine if the mutation is

maintained.

(4) STATISTICAL ANALYSIS:

Essentially, the statistical analysis will involve comparison of the frequency (either singly or in combination) of cyclin D1 and c-erbB-2 overexpression and p53 protein accumulation and/or gene mutation in the cases and controls, using conditional logistic regression with multiple controls per case (101). The association between these genetic changes and factors which are thought to be involved in the etiology of BBD and breast cancer (e.g., reproductive, menstrual, and dietary factors, as well as BBD histology) will be examined, as will the association of the latter variables with risk of progression to breast cancer. Where appropriate, odds ratio estimates for the association between cyclin D1 and c-erbB-2 overexpression and p53 protein accumulation with or without p53 gene mutation and risk of breast cancer will be adjusted for these other factors (i.e., for risk factors associated both with the genetic changes of interest and with risk of progression to breast cancer). To the extent possible, we will examine these associations by interval between the date of diagnosis of benign breast disease and the subsequent development of breast cancer.

Further analyses will be directed towards within-individual comparisons of cyclin D1 and c-erbB-2 overexpression and p53 in BBD and breast cancer. One possible interpretation of any changes which are found to be common to both conditions will be that they contribute to the development of breast cancer rather than arise as a consequence of it.

RESULTS

We have updated the data base with respect to identifying details of the remaining individuals in the cohort with benign breast disease.

We are requesting paraffin blocks from a total of 176 hospitals. To date forty-three laboratories have responded and we have received 475 blocks. Followup phone calls are being made to the lab director, or their designate, of the hospitals/laboratories from which there has been no response. The details of the blocks as they are received are being entered into the data base.

The conditions for immunohistochemical detection of cyclin D1 were developed. Cyclin D1 immunostaining of tissue sections from the existing blocks of benign breast disease has been completed. Positive immunostaining of benign breast disease was seen, however histological review of all of the slides, coding and data entry of the results are ongoing and not yet complete so there are no results yet.

In the first year of this grant, the technical objectives 1 and 2 (tasks 1, 2, and 3)as detailed above have been initiated within the timelines indicated in the statement of work in the grant proposal.

However, technical objective 3 (tasks 4 and 5) in the statement of work in the grant proposal has not been accomplished because of circumstances beyond our control. This task involves extension of the ongoing project by the addition of more cases and their controls. The cases are identified by the linkage of the NBSS database to the National Cancer Incidence Reporting System. There was a delay at Statistics Canada and the linkage has just been completed. Currently they are verifying the breast cancer diagnoses and should be able to provide the information necessary to identify the additional cases by December 1997. At

that time we will begin the c-erbB-2, p53 and cyclin D1 immunostaining and molecular studies as described.

CONCLUSIONS

As the work is ongoing there are as yet no conclusions.

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